



How I curate: applying American Society of Hematology-Clinical Genome Resource Myeloid Malignancy Variant Curation Expert Panel rules for *RUNX1* variant curation for germline predisposition to myeloid malignancies

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ABSTRACT

The broad use of next-generation sequencing and microarray platforms in research and clinical laboratories has led to an increasing appreciation of the role of germline mutations in genes involved in hematopoiesis and lineage differentiation that contribute to myeloid neoplasms. Despite implementation of the American College of Medical Genetics and Genomics and Association for Molecular Pathology 2015 guidelines for sequence variant interpretation, the number of variants deposited in ClinVar, a genomic repository of genotype and phenotype data, and classified as having uncertain significance or being discordantly classified among clinical laboratories remains elevated and contributes to indeterminate or inconsistent patient care. In 2018, the American Society of Hematology and the Clinical Genome Resource co-sponsored the Myeloid Malignancy Variant Curation Expert Panel to develop rules for classifying gene variants associated with germline predisposition to myeloid neoplasia. Herein, we demonstrate application of our rules developed for the *RUNX1* gene to variants in six examples to show how we would classify them within the proposed framework.

Introduction

Germline mutations in genes involved in hematopoiesis and lineage differentiation predispose patients to myeloid neoplasia with or without thrombocytopenia. The broad adoption of next-generation sequencing and microarrays in the clinical laboratory has expanded our knowledge of germline contribution to myeloid neoplasia. Drazer *et al.* reported that in six of 24 patients with myeloid neoplasia, presumed somatic variants in *DDX41*, *GATA2* and *TP53* were of germline origin.¹ Similarly, Churpek *et al.* showed that 29% of acute myeloid leukemia (AML)/myelodysplastic syndrome (MDS) kindreds with a positive family history carried a variant in one of 12 genes associated with germline predisposition to hematopoietic malignancies, including *FANCA*, *GATA2*, *RUNX1*, and *SBDS*.² To date, more than 65 genes have been associated with a predisposition to hematologic malignancies.³ Recognizing the contribution of germline variation toward myeloid neoplasia, the ‘WHO classification of Tumors of Hematopoietic and

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Lymphoid Tissues' incorporated the classification of myeloid neoplasia with germline predisposition in their 2016 revised edition.^{4,5}

In parallel, clinical laboratories are increasingly offering broad next-generation sequencing-based tests for patients with myeloid neoplasia for somatic testing, and will readily detect germline variants, if present in a patient. While there is increased clinical awareness of the potential for these germline variants to contribute to a patient's disease, there are often insufficient data in the literature to definitively classify whether a detected variant is contributing to the patient's phenotype.^{6,7} For example, familial platelet disorder with predisposition to AML (FPD/AML) is an autosomal dominant disorder in which germline mutations in *RUNX1* result in thrombocytopenia, platelet functional and/or ultrastructural defects, and/or susceptibility to hematologic malignancies commonly including MDS, AML, and other malignancies⁸⁻¹¹ (Table 1). ClinVar (<https://www.ncbi.nlm.nih.gov/clinvar/>) is a database repository of clinically actionable genomic variants^{12,13} that currently lists 325 germline *RUNX1* variants deposited by clinical laboratories. More than half of these variants are currently reported as being of uncertain significance.

Worldwide, most clinical laboratories follow the 2015 American College of Medical Genetics and Genomics (ACMG) and the Association for Molecular Pathology (AMP) guidelines for sequence variant interpretation.¹⁴ In this framework, germline variants are classified using a five-tier system: benign (BEN), likely benign (LBEN), variant of uncertain significance (VUS), likely pathogenic (LPATH) and pathogenic (PATH). During sequence variant interpretation, laboratories systematically review the supporting criteria of a genomic variant, such as: minor allele frequencies (MAF), computational predictions, functional experiments and segregation with disease in order to determine the five-tier classification.^{14,16}

Although the ACMG/AMP guidelines provide a comprehensive framework for sequence variant interpretation, the high rate of VUS and curation discrepancies continue to be an impediment to accurate clinical annotation and interpretation of genomic variants.^{6,7} To encourage genomic and phenotypic data sharing, and engage experts in consensus-driven variant interpretation, the Clinical Genome Resource (ClinGen) convened Variant Curation Expert Panels (VCEP) to develop gene- and disease-specific modifications of the original guidelines and provide expert-reviewed variant classification for depositing into ClinVar (*Online Supplementary Figure S4*).¹⁷ In 2018, the American Society of Hematology (ASH) sponsored a ClinGen Myeloid Malignancy Variant Curation Expert

Panel (MM-VCEP), composed of 34 international members, who started working on gene- and disease-specific rules for *RUNX1* as the first of several genes conferring predisposition to myeloid malignancies (*Online Supplementary Figure S1A*). After designing, modifying and testing the preliminary *RUNX1* rules on 52 pilot variants, which improved classification in 33% VUS or variants with conflicting interpretations (CONF), MM-VCEP-specified ACMG/AMP rules were approved by the ClinGen oversight committee and efforts to curate variants to ClinVar using the Variant Curation Interface have commenced (*Online Supplementary Figure S1B*).¹⁸ This pilot effort resulted in one variant being upgraded to PATH, two variants being upgraded to LPATH, and three variants being downgraded to LBEN. ClinGen's website contains the MM-VCEP variant classification recommendations and any subsequent modifications to these codes over time (<https://www.clinicalgenome.org/affiliation/10034/>).

Herein, we demonstrate the application of *RUNX1*-specific rules (Table 2) to classify nine representative *RUNX1* variants in six examples (Table 3) while reviewing phenotypic criteria for FPD/AML and summarizing molecular and functional roles of *RUNX1*.

Example 1. Early nonsense variants, (p.Arg204Ter) (PATH with PVS1, PM2, PS4 supporting, and PP1)

A 50-year old female with new pancytopenia was referred to a hematology service. A bone marrow biopsy showed hypocellularity with severe trilineage dysplasia and 12% blasts, diagnostic of MDS with excess blasts (MDS-EB-2). Further investigation showed pathogenic variants in *RUNX1* (NM_001754:c.610C>T, (p.Arg204Ter)), *BCOR* and *ASXL1* with a normal karyotype. The medical history was positive for thrombocytopenia (baseline 70-120x10⁹/L) and a propensity to excessive bleeding after tooth extractions. The family history was positive for two sons with persistent thrombocytopenia (baseline 50-100x10⁹/L) not otherwise explained and a granddaughter with thrombocytopenia and MDS with monosomy 7 (Figure 1). During the initial assessment, an increase in lactate dehydrogenase and the peripheral blast count were noted. A second marrow biopsy confirmed transformation into AML with 40% blasts. The patient underwent induction chemotherapy without achieving remission and clofarabine bridging for unrelated stem cell transplantation. During conditioning, the patient developed sepsis with Gram-negative bacteria and died shortly afterwards. Since she had a remarkable personal and family history pointing towards a germline predisposition syndrome, a skin biopsy was performed at the time of the diagnosis of MDS, and DNA testing from cultured skin

Table 1. Clinical phenotypes of RUNX1 familial platelet disorder and hereditary malignancies.

Clinical and laboratory features	Details	Life-time risk
Hematologic malignancy	Commonly AML or MDS; less frequently T-ALL; and rarely mixed MPN/MDS such as CMML, as well as B-ALL, and hairy cell leukemia	~44%
Thrombocytopenia	Mild to moderate thrombocytopenia with normal platelet size, in the absence of other causes	Most patients
Platelet functional and/or ultrastructural defects	Includes impaired platelet aggregation (particularly in response to collagen and epinephrine) and platelet alpha or dense granule secretion defects	Not known

Adapted from Table 2 from Luo and Feurstein, *et al.*¹⁸ AML: acute myeloid leukemia; ALL: acute lymphoblastic leukemia; MPN: myeloproliferative neoplasms; MDS: myelodysplastic syndrome, CMML: chronic myelomonocytic leukemia.

Table 2. Clinical Genome Resource Myeloid Malignancy Variant Curation Expert Panel-approved rules for *RUNX1* variant interpretation.

ACMG/ AMP CC	Original ACMG/AMP rule summary	Specification	Stand alone	Very strong	Strong	Moderate	Supporting	Comments
BA1	Allele frequency is >5% in ESP, 1000G, or ExAC.	Disease-specific	MAF ≥ 0.0015 (0.15%)	na	na	na	na	(1) The variant is present in any general continental population dataset with a minimum number of 2,000 alleles and variant present in ≥5 alleles.
BS1	Allele frequency is greater than expected for disorder.	Disease-specific	na	na	MAF between 0.00015 (0.015%) and 0.0015 (0.15%)	na	na	(1) The variant is present in any general continental population dataset with a minimum number of 2,000 alleles and variant present in ≥5 alleles. (2) Variant can be classified as likely benign based on BSI alone if there is no contradictory evidence supporting pathogenicity.
BS2	Observed in a healthy adult individual for a recessive (homozygous), dominant (heterozygous), or X-linked (hemizygous) disorder, with full penetrance expected at an early age.	na	na	na	na	na	na	FPD/AML patients display incomplete penetrance and the average age of onset of hematologic malignancies is 33 years.
BS3	Well-established <i>in vitro</i> or <i>in vivo</i> functional studies show no damaging effect on protein function or splicing.	Gene-specific, strength	na	na	(1) Transactivation assays demonstrating normal transactivation (80-115% of wt) AND (2) data from a secondary assay demonstrating normal function.	na	Transactivation assays demonstrating normal transactivation (80-115% of wt).	See PSS (1) and (2)
BS4	Lack of segregation in affected members of a family.	General rec	na	na	Applied when seen in ≥2 informative meioses.	na	na	This code should only be applied for genotype-positive, phenotype-negative (with sufficient laboratory evidence) family members.
BP1	Missense variant in a gene for which primarily truncating variants are known to cause disease.	na	na	na	na	na	na	FPD/AML is caused by both pathogenic missense and truncating variants.
BP2	Observed <i>in trans</i> with a pathogenic variant for a fully penetrant dominant gene/disorder or observed <i>in cis</i> with a pathogenic variant in any inheritance pattern.	General rec	na	na	na	na	Per original ACMG/AMP guidelines.	BP2 can also be applied if the variant is detected in a homozygous state.
BP3	In-frame deletions/insertions in a repetitive region without a known function.	na	na	na	na	na	na	<i>RUNX1</i> does not contain a repetitive region without known function.
BP4	Multiple lines of computational evidence suggest no impact on gene or gene product.	General rec	na	na	na	na	Per original ACMG/AMP guidelines.	BP4 should be applied for missense variants if all of the following apply: (1) REVEL score <0.15, (2) SSF and MES predict either an increase in the canonical splice

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ACMG/ AMP CC	Original ACMG/AMP rule summary	Specification	Stand alone	Very strong	Strong	Moderate	Supporting	Comments
BP5	Variant found in a case with an alternate molecular basis for disease.	na	na	na	na	na	na	site score or a decrease of the canonical splice site score by no more than 10% and (3) no putative cryptic splice sites are created. BP4 should also be applied for synonymous, intronic and non-coding variants for which SSF and MES predict either an increase in the canonical splice site score or a decrease of the canonical splice site score by no more than 10% and no putative cryptic splice sites are created.
BP6	Reputable source recently reported variants as benign, but data are not available for laboratories to perform independent evaluations.	na	na	na	na	na	na	In rare circumstances, a patient can carry two variants in genes predisposing to hematologic malignancies.
BP7	A synonymous variant for which splicing prediction algorithms predict no impact to the splice consensus sequence nor the creation of a new splice site AND the nucleotide is not highly conserved.	General rec	na	na	na	na	Per original ACMG/AMP guidelines. BP7 cannot be applied in combination with PP3.	According to SVI recommendations. Also applicable to intronic/non-coding variants at or beyond positions +7/-21 for which (1) SSF and MES predict either an increase in the canonical splice site score or a decrease of the canonical splice site score by no more than 10% and no putative cryptic splice sites are created AND (2) evolutionary conservation prediction algorithms predict the site as not conserved (e.g. PhyloP score <0.1 or the variant is the reference nucleotide in one primate and/or three mammal species.).
PVS1	Null variant in a gene for which LOF is a known mechanism of disease.	Gene-specific, strength	na	na	Per modified <i>RUNX1</i> PVS1 decision tree for SNV, indels and CNV and table of splicing effects.	na	na	<i>RUNX1</i> LOF variants are a common mechanism of disease in FPD/AML. Three major isoforms (A, B, C) are expressed by use of two promoters and alternative splicing. C-terminal variants not predicted to undergo NMD are classified as PVS1_strong, deletions of exons 2 and 3, presumably only affecting <i>RUNX1</i> isoform 1C, meet PVS1_moderate.
PS1	Same AA change as a previously established pathogenic variant regardless of nucleotide change.	Strength	na	na	Same AA change as a previously established pathogenic variant regardless of nucleotide change.	Same AA change as a previously established likely pathogenic variant regardless of nucleotide change.	na	(1) RNA data or agreement in splicing predictors show no splicing effects (SSF and MES predict either increase in canonical splice site score or decrease of canonical splice score by no more than 10% and no putative splice site are created). (2) The previously established PATH/LPATH variant must be asserted pathogenic/likely pathogenic based on MM-VCEP rules for <i>RUNX1</i> before this rule can be applied.
PS2	De novo (maternity and paternity confirmed) in a patient with the disease and no family history.	Disease-specific, strength	na	na	≥ 2 proven <i>de novo</i> occurrences (maternity)	≥ 2 proven <i>de novo</i> occurrences (maternity)	1 proven <i>de novo</i> occurrence (maternity)	(1) No family history is defined as: absence of the variant and any of the <i>RUNX1</i> -phenotypic criteria in first- and/or second-degree relatives. (2) The proband must exhibit at least one phenotypic FPD/AML criterion. (3) The

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ACMG/ AMP CC	Original ACMG/AMP rule summary	Specification	Stand alone	Very strong	Strong	Moderate	Supporting	Comments
PS3	Well-established <i>in vitro</i> or <i>in vivo</i> functional studies supportive of a damaging effect.	Gene-specific, strength	na	na	Transactivation assays demonstrating altered transactivation (<20% of wt, and/or reduced to levels similar to well-established pathogenic variants such as Arg201Gln or Arg166Gln) AND data from a secondary assay demonstrating altered function. PS3 cannot be applied if the variant meets PVS1. If the variant meets criteria for PVS1_strong and PS3, we recommend either applying PVS1_strong and PS3_moderate or upgrading PVS1_strong to PVS1 without applying PS3.	and paternity confirmed) in patients with the <i>RUNX1</i> -phenotype. Transactivation assays demonstrating altered transactivation (<20% of wt, and/or reduced to levels similar to well-established pathogenic variants such as Arg201Gln or Arg166Gln) secondary assays demonstrating altered function.	and paternity confirmed) in a patient with the <i>RUNX1</i> -phenotype. Transactivation assays demonstrating enhanced transactivation (>115% of wt).	maximum allowable strength by combining PS2 and PM6 criteria is to apply one moderate or two supporting rules. (1) Transactivation assays should include wt and known pathogenic controls, as well as co-expression with <i>CBFB</i> . Promoter sequences of <i>CSF1R</i> (M-CSF-R), <i>PFA</i> , <i>C-FMS</i> and <i>GZMB</i> , containing consensus <i>RUNX1</i> binding sites have been used for transactivation assays. (2) The following secondary assays have been performed: EMSA and yeast hybrid assays (decreased DNA-binding affinity), co-IP, FRET and affinity assays (diminished heterodimerization ability with <i>CBFB</i>), IP and WB with cell fractionation (abnormal cellular localization), colony-forming assays (reduced colony-forming potential), xenotransplantation experiments (abnormal function of mutant <i>RUNX1 in vitro</i>). (3) PS3 can also be applied for evidence of very low or abnormal mRNA/protein expression of the variant allele as a functional consequence of a null variant or incorrect mRNA/protein products.
PS4	The prevalence of the variant in affected individuals is significantly increased compared to the prevalence in controls.	Disease-specific, strength	na	na	≥ 4 probands meeting <i>RUNX1</i> -phenotypic criteria.	2-3 probands meeting <i>RUNX1</i> -phenotypic criteria.	1 proband meeting <i>RUNX1</i> -phenotypic criteria.	The affected individual has to fit at least one of the <i>RUNX1</i> -phenotypic criteria AND variant has to be either absent from gnomAD (overall population) or only present once.
PM1	Located in a mutational hot spot and/or critical and well-established functional domain without benign variation.	Gene-specific, strength	na	na	na	Variant affecting one of the following 13 hotspot residues: Arg107, Lys110, Ala134, Arg162, Arg166, Ser167, Arg169, Gly170, Lys194, Thr196, Asp198, Arg201,	Variant affecting one of the other AA residues 105-204 within the RHD.	The RHD (AA 77-204) has been established to be a highly conserved DNA-binding domain without any benign variation in ClinVar. No germline pathogenic variants have been reported in residues in the region (AA 77-104) to date. The AA range under PM1 supporting may be expanded in the future to other parts of the protein if more evidence emerges.

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ACMG/AMP CC	Original ACMG/AMP rule summary	Specification	Stand alone	Very strong	Strong	Moderate	Supporting	Comments
						Arg204.		
PM2	Absent from controls.	General rec	na	na	na	Per original ACMG/AMP guidelines.	na	Variant must be completely absent from all population databases. The mean coverage of <i>RUNX1</i> in the population database used should be at least 20x.
PM3	For recessive disorders, detected <i>in trans</i> with a pathogenic variant.	na	na	na	na			FPD/AML is inherited in an autosomal dominant manner.
PM4	Protein length changes due to in-frame deletions/insertions in a non-repeat region or stop-loss variants.	Gene-specific, strength	na	na	na	In-frame deletion/insertion impacting at least one of the 13 hotspot residues Arg107, Lys110, Ala134, Arg162, Arg166, Ser167, Arg169, Gly170, Lys194, Thr196, Asp198, Arg201, Arg204	Other in-frame deletion/insertion impacting residues 105-204 within the RHD.	see PMI
PM5	Missense change at AA residue where a different missense change determined to be pathogenic has been seen before.	Strength	na	na	Missense change at the same residue where a different missense change previously been determined to be pathogenic. PM5_strong cannot be applied together with PMI.	Missense change at the same residue where a different missense change previously been determined to be pathogenic.	Missense change at the same residue where a different missense change previously been determined to be likely pathogenic.	see PSI
PM6	Assumed <i>de novo</i> (but without confirmation of maternity and paternity) in a patient with the disease and no family history.	Disease-specific, strength	na	na	na	≥4 assumed <i>de novo</i> occurrences (without confirmation of maternity and paternity) in patients with the <i>RUNX1</i>	2 or 3 assumed <i>de novo</i> occurrences (without confirmation of maternity and paternity) in patients with the <i>RUNX1</i>	see PS2

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ACMG/ AMP CC	Original ACMG/AMP rule summary	Specification	Stand alone	Very strong	Strong	Moderate	Supporting	Comments
PP1	Co-segregation with disease in multiple affected family members.	Disease-specific, strength	na	na	≥ 7 meioses observed within one or across multiple families.	phenotype. 5 or 6 meioses observed within one or across multiple families.	phenotype. 3 or 4 meioses observed within one or across multiple families.	(1) Affected individuals show at least one of the <i>RU/X1</i> -specific phenotypic criteria. (2) Only genotype and phenotype positive individuals and obligate carriers are counted. (3) Demonstration of co-segregation in multiple families is not required since many <i>RU/X1</i> variants are unique and only occur in one family. Missense constraint z-score for <i>RU/X1</i> is <3.09.
PP2	Missense variant in a gene that has a low rate of benign missense variation and where missense variants are a common mechanism of disease.	na	na	na	na	na	na	
PP3	Multiple lines of computational evidence support a deleterious effect on the gene or gene product.	General rec	na	na	na	na	Per original ACMG/AMP guidelines.	(1) PP3 should be applied for missense variants with a REVEL score of >0.75 (2) PP3 should be applied for missense or synonymous variants if the variant alters the last three bases of an exon preceding a donor splice site or the first three bases of an exon following a splice acceptor site and the predicted decrease in the score of the canonical splice site (measured by both MES and SSF) is at least 75% regardless of the predicted creation/presence of a putative cryptic splice site. (3) PP3 should also be applied for intronic variants (in introns 4-8) located in reference to exons at positions +3 to +5 for splice donor sites or -3 to -5 for splice acceptor sites for which the predicted decrease in the score is at least 75% (measured by both MES and SSF) regardless of the predicted creation/presence of a putative cryptic splice site. (4) PP3 cannot be applied for canonical splice site variants.
PP4	Patient's phenotype or family history is highly specific for a disease with a single genetic etiology.	na	na	na	na	na	na	FPD/AML does not exhibit a highly specific phenotype and there is substantial genetic heterogeneity.
PP5	Reputable source recently reports variant as pathogenic, but the evidence is not available to the laboratory to perform an independent analysis.	na	na	na	na	na	na	According to SVI recommendations.

Adapted from Table 1 of Luo and Feunstein *et al.*¹⁹ ACMG: American College of Medical Genetics; AMP: Association for Molecular Pathology; CC: criteria code; ESP: Exome Sequencing Project; 1000G: 1000 Genome Project; ExAC: Exome Aggregation Consortium; MAF: minor allele frequency; na: not applicable; FPD/AML: familial platelet disorder with predisposition to acute myeloid leukemia; rec: recommendation; SSF: splice site finder; MES: MaxEntScan; SVI: ClinGen Sequence Variant Interpretation Working Group; LOF: loss-of-function; SNV: single-nucleotide variant; CNV: copy number variant; NMD: nonsense-mediated decay; AA: amino acid; MMA/CEP: Myeloid Malignancy Variant Curation Expert Panel; wt: wildtype; EMSA: electrophoretic mobility shift assay; IP: immunoprecipitation; FRET: fluorescence resonance energy transfer; IF: immunofluorescence; WB: western blot; gnomAD: Genome Aggregation Database; RHD: Runt homology domain.

fibroblasts later confirmed the germline, nonsense *RUNX1* variant. Her two sons and granddaughter also tested positive for the *RUNX1* variant (Figure 1).

Similar to this case, most patients with FPD/AML will have a characteristic phenotype (Table 1) including mild to moderate thrombocytopenia with normal platelet size, platelet α or dense granule secretion defects and impaired

platelet aggregation, particularly in response to collagen and epinephrine as well as a predisposition to hematologic malignancies. Although there is variability in disease onset in FPD/AML,³ development of a hematologic malignancy is common with a lifetime risk of ~44%: AML and MDS are common, other malignancies occur less frequently (Table 1).^{19,23} FPD/AML has a high but incomplete

Table 3. Summary of *RUNX1* variant examples with application of the Myeloid Malignancy Variant Curation Expert Panel criteria.

Example No.	<i>RUNX1</i> variant NM_001754 (isoform C)	ClinVar Assertion	Criteria	MM-VCEP classification
1	c.610C>T (p.Arg204Ter)	PATH	PVS1, PM2, PS4_supporting, PP1	PATH
2	c.314A>C p.(His105Pro) c.315C>A p.(His105Gln)	VUS	PM2, PP3, PS4_supporting, PM1_supporting, PM5_supporting PS3, PM2, PP3, PM1_supporting	LPATH LPATH
3	c.253C>A p.(His85Asn)	CONF: OMIM: PATH Invitae: VUS	BS1, BS3, PP3	LBEN
4	c.508+3delA c.444C>T p.(Thr148=)	PATH Illumina: VUS Invitae: LBEN	PS3, PP1_strong, PM2, PP3, PS4_supporting BP4, BP7	PATH LBEN
	c.1257G>A p.(Val419=)	VUS		VUS
5	Copy number variant, deletion of exon 2		PS4, PP1_strong, PM2, PVS1_moderate	PATH
6	c.1118C>A (p.Ser373Ter)		PVS1_strong, PM2, PS4_supporting	LPATH

The five-tier ClinVar classification: PATH (pathogenic), LPATH (likely pathogenic), VUS (variant of uncertain significance), LBEN (likely benign), BEN (benign); CONF (conflicting interpretations in ClinVar); criteria from Luo and Feurstein *et al.*¹⁸

***RUNX1* NM_001754:c.610C>T, (p.Arg204Ter)**

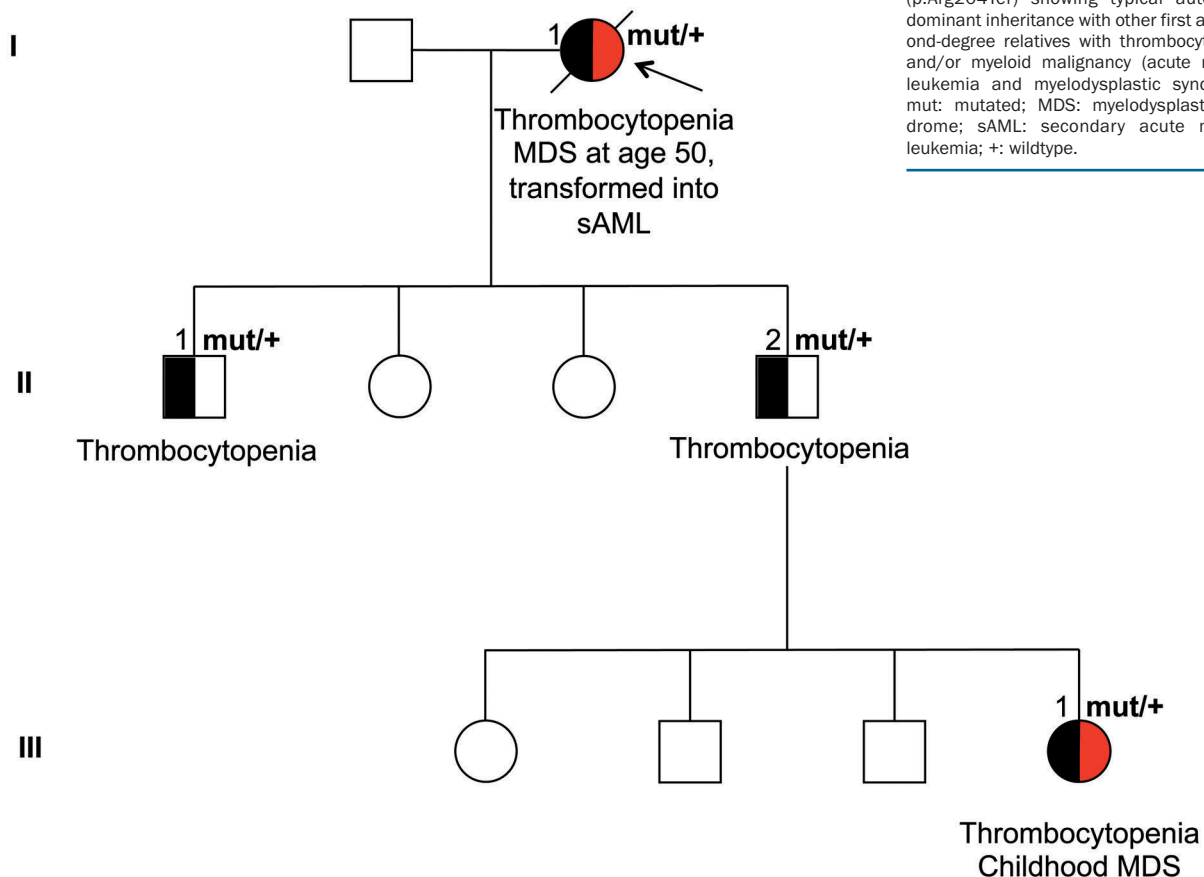


Figure 1. Family pedigree of a patient with acute myeloid leukemia, example 1. Germline *RUNX1* NM_001754:c.610C>T, (p.Arg204Ter) showing typical autosomal dominant inheritance with other first and second-degree relatives with thrombocytopenia and/or myeloid malignancy (acute myeloid leukemia and myelodysplastic syndrome). mut: mutated; MDS: myelodysplastic syndrome; sAML: secondary acute myeloid leukemia; +: wildtype.

A

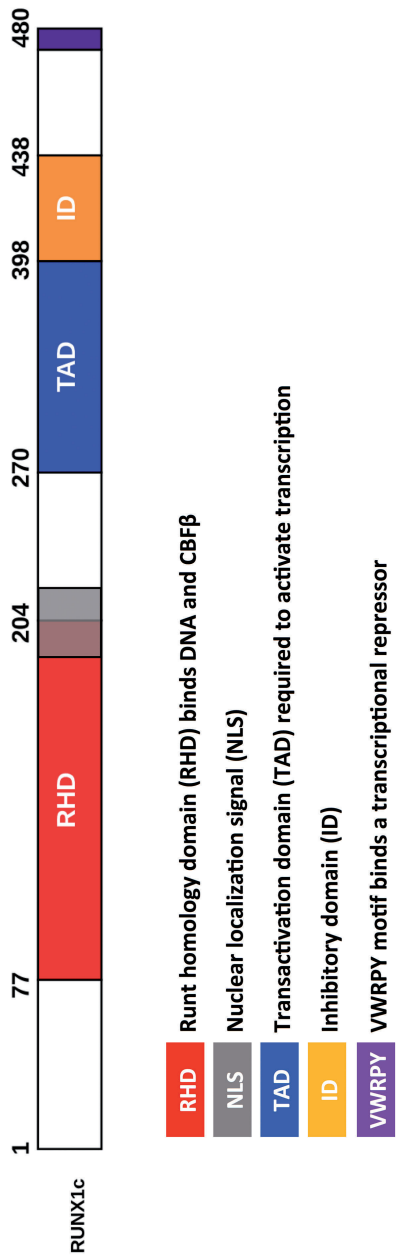
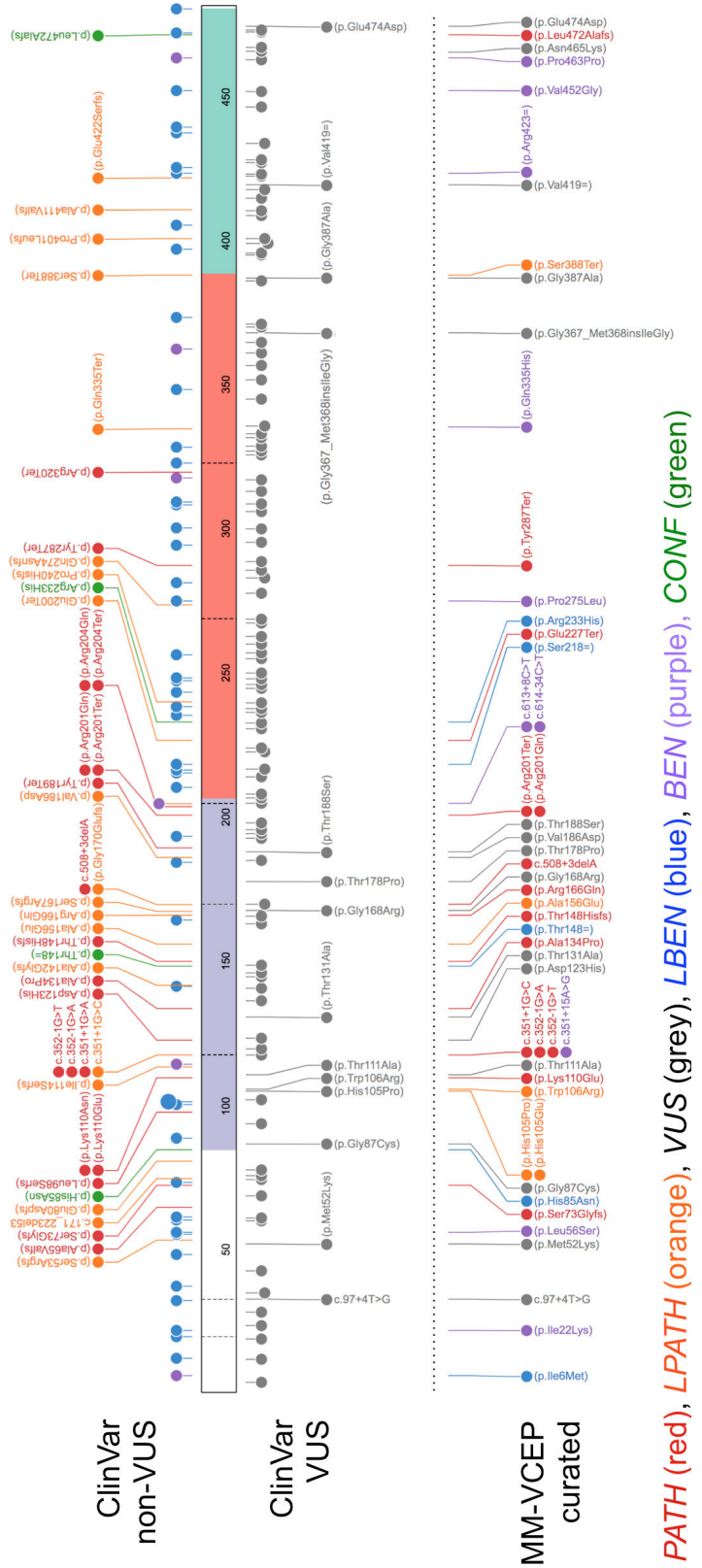


Figure 2. Overview of RUNX1 protein domains, ClinVar-deposited and MM-VCEP curated pilot and COSMIC variants. (A) Schematic of the RUNX1 protein showing its key domains, including the Runt homology domain (RHD); annotation according to MM-VCEP is based on RUNX1 transcript isoform C (NM_001754). (B) Schematic of ClinVar-deposited RUNX1 variants in a one-dimensional line plot showing the current ClinVar five-tier classification; data plotted using *proteinpaint* from St. Jude.org with data from ClinVar (accessed 6/2019); RHD (purple), and ID (green) are highlighted; numbers indicate amino acid; dotted lines indicate exon boundaries. Some ClinVar-deposited variants (in 5' and 3' untranslated regions and large deletions) are not shown. Lollipop representing the variants are colored as follows: PATH (red), LPATH (orange), VUS (grey), LBEN (blue), BEN (purple), CONF (green). MM-VCEP pilot variants²⁸ are shown in the third row. (continued on next page)

RUNX1 amino acid numbering uses transcript isoform C (NM_001754) as the preferred one for annotation according to MM-VCEP

B



penetrance, with several affected individuals reported to have normal platelet counts or function.^{19,24} The nonsense mutation in this patient (p.Arg204Ter) is predicted to lead to nonsense-mediated decay of the *RUNX1* mRNA transcript. *RUNX1* is located on the long arm of chromosome 21 and is translated into three major isoforms, designated *RUNX1A*, *RUNX1B*, and *RUNX1C*, by using two different promoters and alternative splicing. All transcripts are expressed during hematopoietic differentiation and/or maintenance of normal bone marrow function.²⁵⁻³² For variant annotation, the MM-VCEP utilizes the longest isoform, *RUNX1C* (NM_001754), as the default transcript, which includes all key domains such as the 128 amino acid (AA) long RUNT homology domain for DNA binding (RHD, AA 77-204), transactivation domain (TAD), inhibitory domain (ID) and the transcriptional repressor binding motif (VWRPY) and is most often used by clinical laboratories for *RUNX1* variant curation (Figure 2A). Germline variants have been reported throughout the gene in ClinVar with the majority currently classified as VUS (Figure 2B). The *RUNX1* protein heterodimerizes through its RHD with CBF β to form a master hematopoietic transcription factor (Figure 2C), which is essential for proliferation and differentiation of hematopoietic stem and progenitor cells, especially in the case of megakaryocytic differentiation.³³⁻³⁵ Somatic mutations commonly occur in *RUNX1* (Figure 2D). According to *RUNX1*-specific criteria (Table 2),¹⁸ the MM-VCEP applied the following codes (Table 3): PVS1 (nonsense variant predicted to undergo nonsense-mediated decay), PM2 (absence in all population databases), PS4_supporting (one proband meeting at least one of the *RUNX1* phenotype criteria), PP1 (co-segregation with disease in the family, three meioses) and arrived at a consensus classification of PATH.

Example 2. Missense variants, p.(His105Pro) (LPATH with PM2, PP3, PS4_supporting, PM1_supporting, and PM5_supporting) and p.(His105Gln) (LPATH with PS3, PM2, PP3, and PM1_supporting)

Missense mutations in *RUNX1* commonly occur in the RHD in somatic and germline contexts.³⁶⁻³⁸ Of 325 *RUNX1* ClinVar variants 122 (37.5%) are missense, and currently in ClinVar, none in the RHD has been classified as BEN or LBEN (Figure 2C). When a novel missense variant is identified which has not been established as PATH or LPATH, it can be difficult to know whether the given change will affect protein function and explain the patient's phenotype. For example, two *RUNX1* missense variants in the RHD (NM_001754:c.314A>C, p.(His105Pro); and NM_001754:c.315C>A, p.(His105Gln)) were considered during the pilot variant analysis. The former was initially classified as a VUS in ClinVar (Figure 2B), but subsequently revised to LPATH upon MM-VCEP review (Figures 2B, 3A and 4). The conclusion of the LPATH assertion is based on the codes applied for this variant: PM2, PP3, PS4_supporting, PM1_supporting, and PM5_supporting (Table 3). Since the variant is completely absent from population databases, the MAF code PM2 is applied. For *in-silico* evaluation of missense variants, the MM-VCEP recommends using REVEL, a meta-predictor that combines 13 individual tools with high sensitivity and specificity, which has demonstrated the highest performance compared with individual tools or other ensemble methods.³⁹⁻⁴¹ The computational prediction code PP3 is applica-

ble to the p.His105Pro variant due to a high REVEL score of 0.953 (MM-VCEP defined >0.75 as the cutoff). The ClinVar submitter (SCV000807773.1) provided us with the patient's clinical data from their laboratory and the proband met at least one of the *RUNX1* phenotype criteria (Table 1) which qualified for PS4_supporting. This example emphasizes the critical value of sharing internal laboratory data. There is only one meiosis in this family which is lower than the three required for the segregation code PP1. The MM-VCEP defined 13 residues in the RHD as the mutational hotspots for the PM1 code. In addition, variants in other parts of the RHD (AA 105-204) can have a reduced strength-level resulting in application of PM1_supporting. The last code PM5_supporting is applied on the p.(His105Pro) variant, because a different missense change p.(His105Gln) at the same residue has been classified as LPATH by the MM-VCEP (Table 3).

The codes PM2, PP3, PM1_supporting are also applicable to the p.(His105Gln) variant for the same reasons described. Furthermore, a strong pathogenic code PS3 is applied which contributes a significant weight to the final assertion. Transactivation assays of the p.(His105Gln) variant demonstrate altered transactivation (<20% of wildtype) and secondary assays also indicate altered DNA binding and functional consequences in a mouse model^{42,43} manifested by disturbed myeloid differentiation and induction of a blast crisis or accelerated phase-like phenotype in mice.⁴² These variants highlight the importance of evaluating similar variants and the critical benefit of functional studies showing that variants whose clinical significance were initially uncertain can be subsequently clarified to provide more definitive clinical classification and minimize reporting of VUS. Moreover, these variants demonstrate the value of leveraging the information on one variant to help classify another and data sharing between laboratories (Table 3).

Example 3. Missense variant, p.His85Asn (LBEN with BS1, BS3, and PP3)

RUNX1 NM_001754:c.253C>A, p.(His85Asn) is a missense mutation located within the RHD, but not within the mutational hotspot region (AA 105-204), with conflicting interpretations of pathogenicity in ClinVar (Figure 2B, C). Specifically, this variant had three submissions in ClinVar with two being PATH (submitted in 2002) and one being a VUS (submitted in 2018). The two 2002 submissions are from OMIM, which cited evidence from individual literature sources without a systematic curation process. Osato *et al.* reported an adult patient with AML carrying this variant.⁴⁴ However, the germline nature of the variant was not definitively determined. This variant has also been reported in an infant diagnosed with transient myeloproliferative disorder and Down syndrome whose phenotype does not meet any of the *RUNX1* phenotype criteria.⁴⁵ After analysis and curation by the MM-VCEP using the *RUNX1*-specific classification rules,¹⁸ this variant was re-classified as LBEN, meeting codes BS1 and BS3, despite meeting PP3 (Table 3). According to the penetrance, prevalence and genetic and allelic heterogeneity of *RUNX1*, MM-VCEP refined the *RUNX1* specific MAF threshold for application of BS1 to 0.00015 (0.015%). The highest MAF of the p.His85Asn variant is 0.00043 (8 out of 18,768 alleles) from the East Asian subpopulation in the Genome Aggregation Database (gnomAD) which is higher than the *RUNX1*-

specific BS1 cutoff. Experimental studies have shown that this missense change displays normal transactivation activities (80-114% of wildtype) and does not affect DNA binding, heterodimerization with CBF β or subcellular localization of the RUNX1 protein.^{44,46} Therefore, the strong benign functional evidence code, BS3, is applied. Although this variant disrupts KMT2A binding, which impairs proper H3K4 histone methylation, this is not a qualified functional assay based on the MM-VCEP RUNX1-specific PS3/BS3 rule. Moreover, another well-established BEN variant p.(Leu56Ser) also impairs KMT2A binding.⁴⁶ Likely due to the location of the p.(His85Asn) variant within the RHD (Figure 2B), the REVEL score (0.852) of this variant is higher than the MM-VCEP defined 0.75 cutoff,¹⁸ which results in the variant meeting a conflicting PP3 code. However, combining the BS1, BS3 and PP3 codes, a final assertion of LBEN is made based on a Bayesian classification framework.¹⁵ Given that His85 is located away from binding interfaces in the three-dimensional structure (Figure 3B), it seems

reasonable that variants at this position are LBEN.

This example highlights the value of functional studies in the context of the MM-VCEP variant curation and shows that substantive corrections of variant annotation may occur upon application of ClinGen MM-VCEP rules.¹⁸ Implicit in this process is the expectation that as knowledge about FPD/AML improves with more functional or family data becoming available, the MM-VCEP rules are subject to revision so that annotation of clinical variants will become more accurate (Figure 4). ClinVar variant classifications such as VUS or those with conflicting interpretations may thus evolve to more diagnostic certainty.^{47,48}

Example 4. Synonymous/intronic/non-coding variants, c.508+3delA (PATH with PS3, PP1_strong, PM2, PP3, PS4_supporting), p.Thr148= (LBEN with BP4, BP7) and p.Val419= (VUS with no codes)

RUNX1 variants affecting canonical splice positions ± 1 or 2 at intron-exon boundaries are expected to disrupt

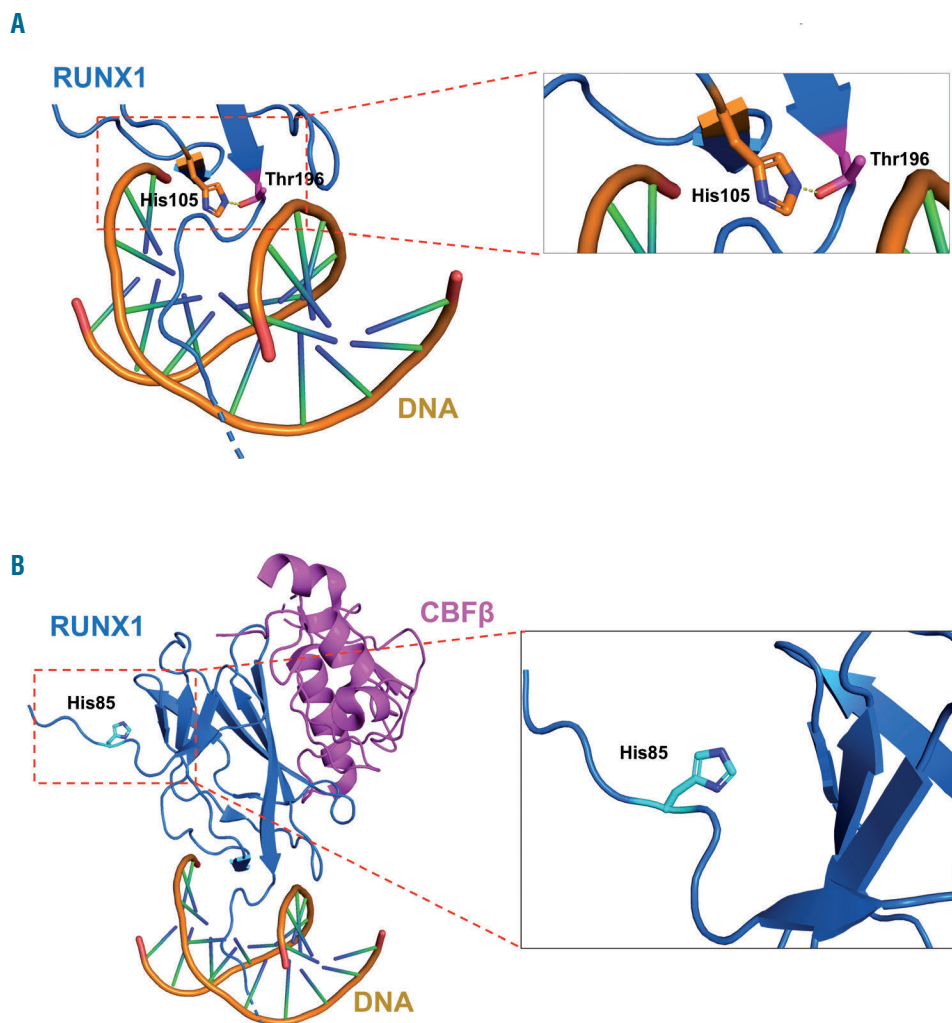


Figure 3. Three-dimensional structure of RUNX1 missense variants His105 and His85 considered as examples 2 and 3. (A) RUNX1 His105 is important functionally due to its location and thus involvement in DNA binding and close interaction with Thr196 by hydrogen bonding. Thr196 is a hotspot residue known to be critical. This structure-function relationship further supports classification of His105 variants as likely pathogenic (LPATH). (B) RUNX1 His85 is located close to the start of the Runt homology domain in a linker region, and is located far from the DNA binding surface. It is not involved in the core β -barrel structure and does not show any interactions, further supporting its classification as likely benign (LBEN). Structure of RUNX1 complexed to DNA and CBF β (<https://www.rcsb.org/structure/1H9D>)⁸² and plotted using PyMOL version 2.3.0.

splicing, leading to protein dysfunction (see *Online Supplementary Table S3* of reference by Luo and Feurstein¹⁸). All of the three canonical splicing site variants in the pilot set were classified as PATH or LPATH. More challenging, however, is the consideration of synonymous/intronic/non-coding variants which may result in cryptic splice site activation, and/or enhancement or repression of adjacent canonical splice sites. For example, the intronic NM_001754:c.508+3delA variant has been reported in a single family with disease segregation (8 meioses, PP1_strong). Several family members were diagnosed with thrombocytopenia, aspirin-like platelet aggregation defects, and dense granule abnormalities.⁴⁹ This variant is absent from population databases (PM2) and both splicing predictors (MaxEntScan and Splice-SiteFinder)^{50,51} predict a significant decrease in the score of the canonical splice site (PP3). Moreover, experimental reverse transcriptase polymerase chain reaction studies (PS3), using RNA derived from two affected family members, were performed and indicate the creation of a novel cryptic splice site 23 nucleotides upstream of the normal splice site resulting in a frameshift p.(Arg162fs*177), and the transcript is predicted to undergo nonsense-mediated decay.⁴⁹ Combining all of these codes, a final assertion of PATH is given by the MM-VCEP (Table 3).

BP7 is a benign code specifically designed to evaluate synonymous/intronic/non-coding variants in the ACMG/AMP framework. BP7 can be applied if computational evidence suggests no impact on splicing, and the nucleotide is not conserved. The ClinVar variant with conflicting interpretations in ClinVar, NM_001754:c.444C>T, p.(Thr148=), has been classified as LBEN by the MM-VCEP using BP7 and the benign *in silico* prediction code, BP4 (Table 3). This nucleotide change is predicted to have no impact on splicing and it is also not conserved (phyloPscore: -4.3832, below the MM-VCEP-specified threshold of <0.1¹⁸). Clinical data from seven individuals with this variant were acquired from the original ClinVar submitter (SCV000761123.1) and revealed that none of the probands met any of the *RUNX1* phenotypic criteria.¹⁸ Currently, only two *RUNX1* variants have been reported to display an abnormal splicing effect as demonstrated by RNA assays.^{11,49} The potential effects of other splicing variants rely solely on *in silico* predictions. Although there is robust effort in consideration of algorithms to predict the effects of splicing variants, these algorithms require further evaluation. Indeed, we know of only limited experimental data within the *RUNX1* gene specifically to test these tools. Accordingly, the synonymous variant, NM_001754:c.1257G>A, p.(Val419=) is predicted to create alternative splice acceptor sites, but is not expected to abolish any existing consensus sites, as it is too far away from either end of the exon. Due to this *in silico* prediction result, none of the PP3/BP4 and BP7 codes can be assigned, and the classification of this variant remains a VUS. Further resolution of the significance of this variant could be obtained through parental testing, and/or RNA-sequencing data.

Example 5. Copy number variants, deletion of exon 2 (PATH with PVS1_moderate, PM2, PS4, and PP1_strong)

Not infrequently, patients with FPD/AML have been reported to have copy number variants resulting in intragenic deletions of *RUNX1*.⁵² As part of our pilot cohort, we evaluated several probands with copy number vari-

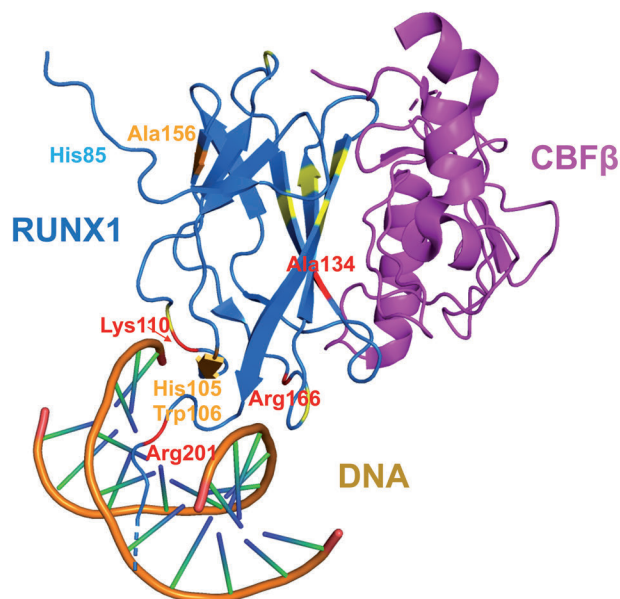


Figure 4. Three-dimensional structure of MM-VCEP-classified *RUNX1* missense variants in the RHD. Variants in the *RUNX1* RHD (blue) are shown highlighting the PATH, LPATH, and LBEN missense variants curated according to ClinGen MM-VCEP rules. PATH (red, n=4), LPATH (orange, n=4) and LBEN (cyan, n=1) variants are found proximate to key interaction domains of *RUNX1* with DNA or its binding partner *CBFβ* (pink). VUS missense variants are shown in yellow (n=7). Non-missense variants and variants outside the RHD are not shown. (PyMOL version 2.3.0; structural data <https://www.rcsb.org/structure/1H9D>⁵³). MM-VCEP: Myeloid Malignancy Variant Curation Expert Panel; RHD: Runt homology domain; PATH: pathogenic; LPATH: likely pathogenic; VUS: variant of uncertain significance; LBEN: likely benign; BEN: benign; CONF: conflicting interpretations in ClinVar.

ants which at a minimum include *RUNX1* exon 2 deletion; data from two cases are shown (Figure 5). The analysis of copy number variants by using next-generation sequencing and/or single nucleotide polymorphism microarrays is particularly challenging because the breakpoints are often not captured in the sequenced regions or the microarray resolution defines only a range for the chromosomal location of the breakpoint, respectively, and thus the nucleotide level breakpoint may remain unknown. It can, therefore, be difficult to know the effect of partial gene/exon deletions, such as if the deletion is in- or out-of-frame, the latter of which may also lead to the introduction of a premature stop codon. Nevertheless, partial or whole gene deletion of *RUNX1* is expected to result in haploinsufficiency of the *RUNX1* protein. Although MM-VCEP rules did not include recommendations for the formal classification of copy number variants,¹⁸ several points should be noted. First, evaluation of the reference *Database of Genomic Variants* (<http://dgv.tcag.ca/dgv/app/home>) shows that copy number variants affecting *RUNX1* do not appear to be frequent.¹⁸ Second, annotation of the specific breakpoints of these intragenic deletions may not always be possible, given that whole genome sequencing is not typically performed. Since contiguous exon deletion is a common pathogenic disease mechanism, it is imperative that laboratories performing germline testing for *RUNX1* use concurrent microarray testing, develop appropriate next-generation sequencing bioinformatics pipelines, or use alternative molecular techniques, such as quantitative polymerase chain reaction and multiplex ligation-dependent probe amplification, to screen for and exclude copy loss,

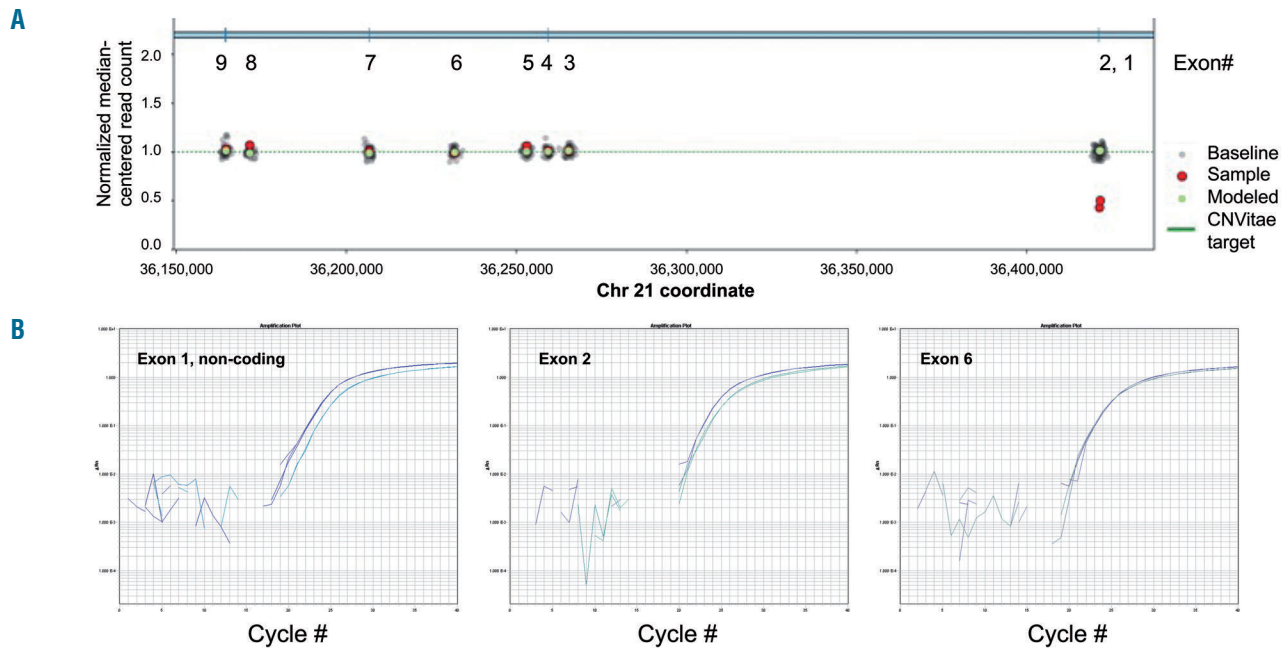


Figure 5. Copy number variant, deletion of exons of *RUNX1*. Testing for germline variants should include evaluation for copy number variants (CNV). (A; B) Clinical data from two different patients showing copy loss of *RUNX1* exons 1 and 2: one proband was identified by next-generation sequencing (A) and the other by single nucleotide polymorphism array analysis (data not shown), and confirmed by quantitative polymerase chain reaction (qPCR) (B). For the latter patient, qPCR confirmation showed heterozygous deletion of exons 1 and 2 with no loss in other exons (exon 6 only shown). CNV are difficult for annotation as breakpoints of the deletion may not be captured and thus, whether the deletion is in- or out-of-frame may not be known without whole genome sequencing. Testing of a germline sample (e.g. fibroblasts) is preferred to blood or bone marrow for CNV evaluation, as somatic copy loss may also occur in the tumor context.

Table 4. Variant details for example 6 from genome and exome sequencing, RNA-sequencing and karyotype analysis.

Structural	<i>KMT2A</i> , NM_005933, 11q23 (chr11:118347001-118353900) partial tandem duplication exons 4-8, predicted to be in-frame
SNVs and indels	<i>IDH1</i> , NM_005896:c.394C>T, p.(Arg132Cys), VAF ~50%
	<i>PHF6</i> , NM_001015877:c.860G>T, p.(Gly287Val), VAF ~ 95%
	<i>RUNX1</i> , NM_001754:c.1118C>A, (p.Ser373Ter), VAF ~50%
Copy number variants	<i>PALB2</i> , heterozygous exon 7 copy loss, NM_02467, chr16p12.2(23637272_23637800)x1 for hg19

Chr: chromosome; VAF: variant allele frequency.

as sequencing for single nucleotide variants and indels alone is insufficient for comprehensive germline evaluation. For the two cases herein (Figure 5), although we do not know the specific breakpoints for each, the common deletion of at least exon 2 allows us to apply the following codes: PVS1_moderate, PM2, PS4 (4 probands: 3 with chronic thrombocytopenia and 1 with AML), PP1_strong (7 meioses) to arrive at a PATH classification (Table 3).

Example 6. Late truncation variant, (p.Ser373Ter) (LPATH with PVS1_strong, PM2, and PS4_supporting)

A 14-year old male with a non-contributory family history presented with malaise, poor appetite, night sweats, and intermittent fever of about 1-month duration, thrombocytopenia ($27 \times 10^9/L$), and subsequent bone marrow biopsy showed AML (PS4_supporting). After whole genome and exome sequencing on paired tumor and germline samples, along with RNA-sequencing (directed for recurrent fusion identification), his leukemia sample was shown to be *RUNX1*-mutated (NM_001754:c.1118C>A, (p.Ser373Ter)), hypodiploid with Y chromosome loss, without chromosomal

fusions, and loss of heterozygosity, but had additional mutations including an intragenic heterozygous deletion of one copy of exon 7 of *PALB2* (Table 4).

Similar to Example 1, this *RUNX1* (p.Ser373Ter) variant is a nonsense mutation; however, it is not predicted to undergo nonsense-mediated decay, but rather is expected to generate a truncated protein without part of the TAD, ID and the VWRPY motif (Figure 2A). From a computational and predictive perspective, a PVS1_strong code is assigned following the PVS1 decision tree for null or truncating variants in *RUNX1*.¹⁸ The variant is absent from the gnomAD and other population databases (with confirmed >20x sequencing coverage at this position in gnomAD). Given the variant's absence from population databases and adequate sequencing coverage of the region, a PM2 code is assigned. Although no additional evidence for the other categories (functional, segregation, *de novo* and allelic data) are available, this variant can be classified as LPATH (PVS1_strong, PM2, PS4_supporting). It is of interest to note that the somatic alterations reported in the diagnostic leukemia sample included partial tandem duplication of *KMT2A* and a single nucleotide varia-

tion in *PHF6*. Alterations in these two genes have been reported as cooperating events seen in leukemias from patients with germline *RUNX1* mutations.^{37,53}

Importantly, if consideration is given to the mutations found in the leukemic cells in isolation, one cannot determine the germline or somatic origin of the variants reported. This is the case for most of the ‘tumor-only’ analyses being performed in many clinical laboratories. Without paired analysis of true germline tissue (e.g. cultured skin fibroblasts), such studies cannot definitively identify germline variants. In this case, the *KMT2A* partial tandem duplication and single nucleotide variation in *PHF6* and *RUNX1* could be tumor-drivers in the AML. However, given the sequencing data, including the variant allele frequency, both the *RUNX1* mutation and the *PALB2* exon 7 intragenic deletion could be germline variants. A detected variant allele frequency approaching 50% or 100% in the tumor may indicate potential germline origin¹ with either an intact wildtype allele or loss of heterozygosity, respectively. However, a high variant allele frequency cannot reliably serve as a proxy for testing of a true germline source. Therefore, if there is concern that a variant could be constitutional, testing of true germline material is critical.¹

Discussion

Kindreds with FPD/AML were first reported by Luddy *et al.* in 1978⁵⁴ and phenotypically well-described as having a bleeding diathesis and myeloid neoplasia by Downton *et al.* in 1985.^{55,56} Subsequent linkage analysis identified *RUNX1* as the candidate gene at chromosome 21q22,¹¹ and mutations were detected in FPD/AML families in 1999.¹¹ Since these initial early reports, routine clinical testing for *RUNX1* gene mutations is now commonplace for the evaluation of somatic and germline disease in patients with myeloid neoplasms and thrombocytopenia.

In general, *RUNX1* variants include single nucleotide variations and indels, such as missense, nonsense, frameshift, and splice site variants, and copy number variations such as whole-gene and intragenic deletions. *RUNX1* is also frequently mutated somatically in AML and often the partner of various translocations resulting in gene fusions, such as t(8;21)(q22;q22) *RUNX1-RUNX1T1*.^{57,58} To date, fusions of *RUNX1* have not been reported in the germline context, and most germline *RUNX1* variants are unique,²⁴ although some have been rarely seen in unrelated families. Given the limited data on rare variants, the clinical annotation of new variants remains challenging. The MM-VCEP was convened by ASH/ClinGen (*Online Supplementary Figure S1*) to develop rules for curating gene variant causing predisposition to myeloid neoplasia (Table 2). In this review, we describe the classification of six variant examples (Table 3) using the gene- and disease-specific rule modifications of the original ACMG/AMP 2015 framework.¹⁴ Several points should be made.

First, it is critical to ensure that genomic testing intended to assess a germline predisposition is performed on a definitive germline sample because malignant hematologic diseases involve the peripheral blood and bone marrow, and somatic variants in these diseases can confound variant interpretation if an inappropriate sample is used.

Here, in keeping with our MM-VCEP rules, cultured skin fibroblasts (gold standard, albeit invasive, costly, and time-consuming), cultured bone marrow mesenchymal stromal cells or DNA from hair roots are appropriate sources.^{59,60} Alternatively, confirmation of the germline nature of a variant can be achieved by demonstrating its presence in two or more related individuals. The possibility of sample contamination by malignant cells is significant and consequently, peripheral blood, bone marrow, saliva, buccal swabs, DNA from paraffin blocks and even fingernails, which can contain monocytes, are inappropriate samples for germline testing. In some institutions, laboratories may accept T cells, enriched via flow cytometry sorting or column-based magnetic cell separation, as a germline sample for testing. It is important to recognize that some somatic alterations may occur early in hematopoietic stem and progenitor cells with multilineage potential to differentiate into T cells,⁶¹ as recent single-cell studies have confirmed.⁶²⁻⁶⁵ Thus, if T cells are used, the possibility that a detected variant may be somatic should still be considered. Once a variant is confirmed to be germline in a proband, however, additional testing for the known variant in related family members can be performed on any tissue source.

Second, we should keep an open mind about disease-causing alleles and the type of variants that may be seen and thus, we advocate for a broad testing approach. For example, in some laboratories, non-coding variants are automatically filtered as part of bioinformatic pipelines and may thus be omitted from subsequent review and interpretation. Recently, however, synonymous variants⁶⁶ in the *GATA2* gene, another gene predisposing to myeloid malignancy, were reported in addition to the known pathogenic deep intronic variants of an enhancer region of *GATA2*.⁶⁷ In *ANKRD26*, variants of the 5' untranslated region cause disease.^{68,69} Furthermore, copy number alterations may not be assessed in somatic tumor testing panels. As diagnosticians, it is important to think broadly when analyzing genomic information for germline pathogenic variants. Given that these are rare diseases, we should not inadvertently exclude disease mechanisms and/or specific classes of mutations. For example, in case 4, some variants may remain as VUS until additional functional or familial segregation data become available for reclassification.^{47,48}

Third, definitive annotation of variants by one institution will likely remain challenging. However, consistent application of MM-VCEP rules with ClinVar data deposition and thus inter-laboratory correspondence can significantly improve the accuracy and consistency of variant curation. In this regard, examples 2, 3, and 5 show how leveraging shared genomic and phenotypic data can be helpful to clarify VUS. We therefore advocate that clinical variant data be deposited into ClinVar. Specifically, laboratories offering germline testing should modify their test requisition forms to indicate that de-identified phenotype and variant data will be deposited into ClinVar as part of ongoing quality assurance and improvement efforts (<https://www.clinicalgenome.org/share-your-data/laboratories/>).^{70,71} Additional details of the ClinVar deposition process are included in *Online Supplementary Figure S1*.

Fourth, *RUNX1* variant curation will improve as more is understood about the disease and gene through functional and family studies. Currently, variant annotation remains a challenging task, because of limited data for

determining the functional effect of a given variant change, despite methods of engineering variants for functional assessment.⁷²⁻⁷⁴ Early studies in Speck's laboratory on *RUNX1* showed the significance of key residues in the RHD of *RUNX1* by performing alanine scanning mutagenesis.^{75,76} However, these early approaches are limited in that not every combination of nucleotide change was explored. By contrast, recent high-throughput functional genomic methods,⁷⁷ known as deep mutational scanning, utilize large-scale approaches to mutate every nucleotide of a gene, permitting one to test the functional consequence of all single nucleotide variations. This has, for example, been recently demonstrated for *BRCA1*.⁷⁸ Additionally, systematic mutagenesis of *PTEN* has provided a wealth of functional data to inform the classification of *PTEN* variants,⁷⁹ in conjunction with published rules developed by the PTEN-VCEP.⁸⁰ In the future, focused functional assays targeting specific VUS¹⁶ and deep mutational scanning of genes should contribute to variant curation to resolve VUS.

Fifth, while functional testing of every given genomic variant is possible, it can be costly and difficult to do for every clinically significant gene. In this regard, family studies can aid in the classification of VUS. By systematically evaluating disease segregation in family members with paired genotyping for a known variant, accurate classification of a given variant can be achieved. For example, a recent study showed that this family-based method for variant classification can resolve a VUS classification more frequently than other traditional approaches can.^{78,81} For rare diseases, such as FPD/AML, detailed pedigree and segregation analyses can be incredibly informative, and clinicians should be encouraged to test family members when possible, seeking help from local genetic counselors and/or geneticists as needed.⁸¹ Hematologists and oncologists need to consistently take a detailed family and genetic history.

Summary

RUNX1 germline mutations associated with FPD/AML are key events in myeloid neoplasms, thrombocytopenia and leukemogenesis and represent a model of a germline gene disorder with pathogenic variants predisposing to myeloid and (to a lesser extent) lymphoid malignancies.³⁶ Providing an accurate clinical and pathologic variant interpretation for genomic variants detected in routine laboratory testing will remain critical for the provision of appropriate clinical care, including genetic counseling for the index patient and their at-risk relatives and donor-selection, in some cases benefiting from stem cell transplantation.

The ClinGen MM-VCEP variant interpretation process requires a detailed understanding of the biological and functional properties of *RUNX1* and disease phenotype. Here, we demonstrate the process for sequence variant

interpretation of six variant examples. By introducing and thus standardizing genomic variant interpretation, we hope to improve patients' care, identify VUS that may benefit from directed research and encourage sharing of internal laboratory data to resolve uncertainty. In doing so, the MM-VCEP rules may ensure optimal insurance coverage for appropriate genomic testing and screening of family members, and ensure appropriate reimbursement for clinical laboratories. Overall, the ASH/ClinGen collaboration resulting in the first set of modified criteria for germline *RUNX1* variants should improve clinical care and recommendations for FPD/AML patients.

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